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(54) **High capacity binary shuttle vector**

(57) The invention provides a high capacity binary shuttle vector having T-DNA region and Ri ori and capable to integrate a large genome fragment in it; a genomic library having the ability to transform a plant, especially monocotyledonous ones; a plant transformed with the high capacity binary shuttle vector; and a method of searching for a useful gene by use of the above vector. The present high capacity binary shuttle vector can introduce a large genome fragment of 10 kb or more, easily, efficiently and stably into a plant, especially monocotyledonous ones under those conditions in which the rearrangement or deletion of these genome fragments does not occur. The invention also provides some other vectors derived from the above Ri ori driven vectors, which can integrate circular genome library plasmids with a lox site, or which can efficiently transform plants with genes of arbitrary expression properties. The invention includes also the assessment method for the functions of genome fragments inserted in the above vectors by complementation assay. In addition, a useful gene or gene complex can be efficiently examined so its isolation is also made easy.

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Description

Field of the Invention

5   The present invention relates to a high capacity binary shuttle vector for plant transformation, etc. Particularly, but not exclusively, the present invention relates to a high capacity binary shuttle vector which may be used for seed plant transformation, particularly monocotyledonous plants which have proved difficult to transform in spite of their economic importance, but it also may be used very powerfully for the complementation test analysis of genome functions and the construction of a genome library for genome function analysis.

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Background of the Invention

     Monocotyledonous plants are more difficult to transform than dicotyledonous plants, in spite of their importance in agriculture. The conventional transformation of these plants has been carried out by various forced introduction

15   methods such as those using gene guns for intact cells, or electroporation or PEG (polyethylene glycol) method for protoplasts.
     However, there are problems in the electroporation method or PEG method in that regeneration from protoplasts to normal plants is difficult, and in case of gene gun, in that expensive facilities are required and a large number of samples can not be processed simultaneously. Furthermore, a large gene fragment of 10 kb or more into monocoty-

20   ledonous plants has not been introduced without its rearrangement.
     Recently, Hiei *et al.* have reported that rice, a typical monocotyledonous crop can be transformed with a pBI system vector and *Agrobacterium* (Hiei *et al.*, Plant Journal, 6:271-282 (1994)). This has made the transformation of rice drastically easier.
     However, the pBI vector used by Hiei *et al.* can stably introduce only about 10 kb DNA into rice. If larger DNA was

25   integrated into such a shuttle vector, the vector could not be stably maintained even in *E. coli.*
     By the recent advances of genome studies, the positional cloning method to isolate a gene, which is based on its positional information on the chromosome map, became the focus of interest as a promising method to isolate a useful gene determining agricultural traits. In order to apply this technique to identify genes causing these traits from the candidate genes selected by using their map information, the trait expression by the gene in the genome fragment

30   must be confirmed by transformation of the fragment to a plant. Therefore, the introduction of larger genome fragments would make such screening of genes much more efficient. Thus, it is desirable to introduce as large a genome fragment as possible.
     Although the binary cosmid method and PEG method are known to introduce genome fragments of 10 kb or larger size into a plant, both of the methods have deficiencies as follows:

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     (1) As for the binary cosmid method, the upper size of a genome fragment to be introduced is limited up to about 20 kb, and pBI system *RK2* is used as replication origin. Therefore, it has been often difficult to stably maintain the plasmid even in *E. coli* into which the plasmid with the chromosome fragment is initially introduced.
     (2) As for the PEG method, regeneration from protoplasts requires a high degree of skill, and often the introduced

40   fragments are severely rearranged making normal complementation by the fragment rather difficult. Therefore, such methods are not sufficiently suited to assay the functions of large genome fragments.

     The present invention seeks to overcome the problems described above. We have now found it possible to introduce a genomic DNA fragment of 10 kb or larger into plants, including higher plants. The technique can also be used

45   with monocotyledonous plants which are difficult to transform and many of them are agriculturally important.
     We have also found it possible to establish an efficient and stable complementation analysis method for a genome region of 10 kb or larger, such regions are very difficult to introduce into plants by conventional methods. By introducing such a large genome fragment efficiently into plants, isolation of the genes controlling various agricultural traits can be made more efficient. We have also found it possible to introduce a large gene complex into a plant as a block to

50   enable them to express complicated traits in plants. Sometimes such a gene complex is located in a group as an operon.
     Thus, the present invention relates to a high capacity binary shuttle vector comprising a T-DNA region, and a *Ri* replication origin.
     The high capacity binary shuttle vector of the present invention is a binary shuttle vector.
     Preferably, said high capacity shuttle vector comprises a gene with a multi-cloning site in the T-DNA region, more

55   preferably the *lacZ* gene.
     Preferably, said high capacity binary shuttle vector comprises at least one antibiotic resistant gene introduced between the *lacZ* gene introduced in the T-DNA region and the border regions of the T-DNA. Said border region is preferably a left border region, and said antibiotic resistance gene is preferably a hygromycin resistant gene.

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Another suitable selection marker using antibiotic resistance is the kanamycin resistance gene which may be used as a selection marker in *E. coli,* and dicotyledonous plants.

Further, said high capacity shuttle vector may integrate fragments with various sizes ranging from a small DNA fragment to a large genome fragment. It has been confirmed that genome fragments with sizes up to at least 40 kb

5   can be efficiently integrated to rice, and even larger sizes may be integrated.

According to one aspect of the present invention there is provided a binary vector comprising a *lox* site in the T-DNA region, a par C gene and an *Ri ori* as replication origin. The vector is capable of integrating a clone plasmid of a circular genome library with a *lox* site by *cre* enzyme, in a host, such as *E. coli* or *Agrobacterium.*

The present invention also provides a binary vector for plant transformation comprising a multi-cloning site sand-

10   wiched between a promoter and a terminator for plants in a T-DNA region, and an *Ri ori* as a replication origin.

The present invention further provides a genomic library having the ability to transform plants. Each clone consti-tuting the genomic library having the ability to transform plants has an insert in the T-DNA region of the high capacity binary shuttle vector of the present invention.

The present invention further provides a complementation method for assessing the function of genes in a genome

15   fragment inserted in a clone of a genome library. This method comprises introducing said plasmid with a genome insert into an *Agrobacterium* cell, and transferring said plasmid into a plant. Furthermore, the present invention provides another complementation method for assessing the function of genes in a genome fragment inserted in a clone of a genome library. This method comprises integrating a component plasmid of a library with a genome insert and a binary vector comprising a *lox* site in its T-DNA region, a par C gene, and an *Ri ori* as replication origin, wherein said vector

20   is capable of integrating a clone plasmid of a circular genome library with a *lox* site by *cre* enzyme, in an *E. coli* or Agrobacterium host; wherein said library is constructed using a circular vector with a *lox* site and *E. coli* as host, introducing said integrated vector into an *Agrobacterium* cell, and then transferring said integrated vector into a plant.

The present invention provides a gene obtained with the complementation method described above.

The present invention also provides a screening method for a useful gene by using the above-mentioned high

25   capacity binary shuttle vector or the library integrating vector.

Furthermore, the present invention provides a plant transformed with said high capacity binary shuttle vector. The plant which may be transformed includes all kinds of plants, preferably seed plants including monocotyledonous plants.

The present invention further provides a method for producing a highly efficient *Agrobacterium* strain with recA⁻ to maintain stably a binary vector with a large insert. This method comprises generating high transformation efficient

30   recA⁻ strains by the site-directed mutation of the recA gene of said strain to transform it to a recA⁻ strain, introducing a homologous recombination to the strains with recA⁺ genes, between the transformed recA⁻ vector and recA⁺ gene in the cell, selecting the recombinant by making a replica of the plate on which the strains are spread, and irradiating the plate to screen a clone which cannot grow under UV irradiation. Alternatively, such recA⁻ strain can be selected by replica after normal mutagenesis and selection under UV irradiation.

35   The present invention thus provides a high transformation efficiency *Agrobacterium* strain with recA⁻, wherein said strain can maintain stably the said high capacity binary vector with large insert, and can transform various plants which are normally recultitrait for transformation.

Various further preferred features and embodiments of the present invention will now be described by way of non-limiting example, in which:

40   FIG. 1 shows the construct of a high capacity shuttle vector pBIGRZ with *Ri ori* and similar vector pBIGZ without *Ri ori.* Construct of the high capacity binary vector pBIGRZ. Arrows of *Ri ori* indicate the direction of the gene and corresponding numbering of the plasmid. Restriction enzymes with the unique cloning site are indicated. RB: right border of T-DNA, LB: left border of T-DNA, *NPTII:* neomycin resistance gene, NP and NT: Promoter and Terminator of nopaline synthetase, *lacZ:* β-galactosidase gene, MCS: multicloning site, P35S: 35S promoter, iGUS: β-glucuronidase

45   gene with an intron, HPT: hygromycin resistance gene, *Ri ori:* replicator origin of *Ri* plasmid.

FIG. 2 shows a construction process for pBIGRZ from pBI121 and pBIHG.

Fig. 3A and 3B show Southern blot analysis of the two genome fragments of about 40 kb, which are introduced into the rice plant by pBIGRZ. A: No deletion or almost no rearrangement of the fragments occurred during transfor-mation as shown by the primary transformants(R₀) as compared with control. This result shows that the genome frag-

50   ments are intactly transferred to plant cells and stably maintained. B: In the first generation of the progeny(R₁), also no deletion nor rearrangement of the fragments were observed. The result shows that the genome fragments integrated into the plant genomes are stably inherited to their progenies.

Fig. 4 shows flowers and panicles of the rice plant into which a human genome fragment of about 40 kb was introduced by pBIGRZ. The rice plant has excellent fertility, and its heads bend down under the weight of seeds.

55   Fig. 5 shows the rice genome library generated using pBIGRZ. They were digested by the restriction enzyme *NotI.* A shows those in which the genome fragments with 45 kb or larger size were introduced. B shows those in which the fragments with 30 to 50 kb were introduced. In A and B, the average size of the introduced fragments were 51 kb and 39 kb, respectively.

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This indicates that a genome library usable for complementation tests may actually be constructed using this vector. The average insert size was obtained by subtracting the vector size which is about 20 kb from the size of each band on the lane. The two lanes on the right side of the gel are size markers.

Fig. 6A and 6B show various inducible vector utilized properties of the high capacity binary shuttle vector of the
5   present invention.

A: a single vector generated by site-directed recombination based on transient cre enzyme expression at lox site. In order to generate this vector, pBRC: one example of a vector to perform complementation test and BAC vector having a genome insert and a lox site was integrated by transient expression of the cre enzyme in host cells.
10 Unintegrated pBRC with BAC is quickly removed when par C sequence is present in it.
B: an example of the vector, pBRPT. This vector is used for introducing a single gene with an expression property from a promoter and terminator, and having a high transformation efficiency of the high capacity vector of the present invention.

15 The high capacity binary shuttle vector of the present invention may be contructed using the following procedure.

(1) Strategy of a binary vector capable of stably maintaining a large genome fragment as an insert (see FIGs. 1 and 2).

A binary vector system complying with the following requirements are used as the base:
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(i) A vector with a T-DNA region, having various genome fragments which are introduced into it as inserts. This T-DNA region can be efficiently introduced into various plant cells.
(ii) A genomic library is constructed easily and stably.
(iii) Plasmid DNA is also prepared easily and E. coli can be used as a host cell.
25 (iv) The constructed plasmid can be easily transduced into Agrobacterium and maintained stably, to make efficient transformation of plants possible.

As described above, the high capacity binary shuttle vector of the present invention can be transduced into Agrobacterium.
30 Agrobacterium tumefaciens is a soil bacterium having a Ti plasmid of about 200 kb in size, and on the Ti plasmid there is a 10 to 20 kb region called T-DNA. When a plant is infected with Agrobacterium, the T-DNA region is excised by the action of a gene product of the vir (virulence) region on the Ti plasmid from Agrobacterium. It is known that the T-DNA thus excised is transferred into a plant cells and finally integrated into their nuclear DNA.
The T-DNA region and vir region may be included in separate vectors. Therefore, it is possible to construct a binary
35 vector consisting of the T-DNA region, at least one origin of replication which can work in E. coli and Agrobacterium and a marker gene only.
The transfer and integration of T-DNA requires only 25-bp border sequences located at both ends of the T-DNA, and none of the other gene groups located in T-DNA is required. The border sequences located at both ends of T-DNA are called left border (LB) and right border (RB), respectively.
40 Various binary shuttle vectors which comply with the above requirements can be used as the base of construction, as long as it can still be used in the mircoorganisms used as the host when it has a proper replication origin, and function as a binary vector. For example, pBI system vectors such as pBI101 and pBI121 are commercially available, and can be conveniently used.
The replication origin used in the pBI binary vector is generally RK2. However, if RK2 is used as the replication
45 origin, often large inserts of 10 kb or larger are not maintained stably in E. coli or Agrobacterium. Further its high copy number may lead to mutual recombination making the plasmid instable. Therefore, the vector should be devised to have high capacity for large inserts and stability in any of the hosts. By introducing Ri as the low copy number replication origin from Rizobium bacteria, the vector was found by us to have high capacity and stability. In this case, the Ri ori may be included in the vector singly or together with RK2.
50 The selection marker for the vector plasmid includes various kinds of antibiotic resistant genes, preferably the kanamycin resistance gene (NPT), hygromycin resistant gene (HPT), gene and so forth are used. These markers can be introduced as required into the plasmid using the known procedure.
Although a basic vector plasmid in the pBI system has only a kanamycin gene as the selection marker in the host plant, hygromycin resistant gene may additionally be introduced. This is because kanamycin does not work well in rice
55 as a s lection marker.
Some restriction sites which interfere the multiple cloning site may be removed or modified. For example, an EcoRI site in said hygromycin resistant gene was modified so as not to be digested with the restriction enzyme.
The genom or DNA fragments to be introduced into said binary vector can be obtained using known methodsfrom

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any kind of organism including various microorganisms, filamentous fungi, protozoan, animals and plants.

Any DNA or genome fragments of at least about 40 kb or more in size can be introduced into the binary vector and selected depending on the requirement. As the transformation of rice with 40 kb genome fragment was done without any difference of efficiency from a few kb of gene, it will be possible to introduce larger fragments without difficulty.

It is preferable to introduce cloning sites in a marker gene to check for the introduction of genome fragment. As the marker genes a *lacZ* (β-galactosidase) gene with cloning sites such as *Hind*III, *Spe*I, *Not*I can be used.

The following genes can be further introduced into the vector, that is, genes to monitor the introduction and expression of the T-DNA in a transformed plant with the binary shuttle vector.

The NPT or HPT genes can be used as such marker genes. β-glucuronidase (GUS) gene is particularly suited to visualize the localized expression sites. Intron-containing GUS (iGUS) is expressed in only eukaryte cells including plant cells, but not in prokaryote cells such as *Agrobacterium*. Therefore by using intron-containing GUS as the expression marker the noise from the contaminating *Agrobacterium* can be eliminated.

However, introduction of many makers can adversely affect the expression of the genes of the interest, such markers in the vector should be kept as few as possible.

(2) Introduction of the genome fragment into the vector and transformation of *E. coli*

(i) Introduction of an desirable DNA fragment into the vector

The vector obtained in (1) above, and fragment DNAs (genome fragments) of suitable size are ligated to the vector using commercially-available ligase, etc. The resulting reaction product is introduced into *E. coli* DH10B cells by electroporation. The cells are then plated onto an LB agar plate containing kanamycin or hygromycin B (50 μg/ml), X-gal and IPTG (isopropyl thiogalactoside). Under usual conditions, the plates are incubated overnight and white colonies formed on the LB agar plate are selected as the transformants in which the above DNA fragment is integrated as an insert.

(ii) Preparation of a genomic library by the transformation vector

Suitable restriction enzymes, most cases *Hind*III were used optionally to digest partially or completely the genomic DNA from plant cells or other materials. The fragments thus obtained are separated based on their sizes by CHEF (contour-clamped hexagonal electric field) gel electrophoresis. The DNA fragments thus obtained are ligated to the vector from (1) above. The resulting products are introduced by electroporation into *E. coli* DH10B cells, and the cells having the above DNA fragment as the insert are plated and selected using the same procedure as in (i) on an LB agar plate containing kanamycin (25 μg/ml) or hygromycin B (50 μg/ml), X-gal and IPTG.

(3) Transfer of the vector from *E. coli* to *Agrobacterium*

The vector with aninsert can be prepared from *E. coli* and can be transferred to *Agrobacterium* by the electroporation method or triparent method (triparent mating method).

(i) Electroporation method

The binary vector with aninsert is recovered from *E. coli* by using the conventional mini-prep method with alkali. If an automatic plasmid extractor is available, a large number of vectors can be extracted efficiently and very easily.

As the recipient *Agrobacterium* strain of the vector , EHA101 is preferable due to its high efficiency. Particularly, rec A strain which lacks recombinant ability induced from high transformation efficiency strains is suitably employed when large fragments are inserted into those cells. Electro-competent cells are prepared by the known method from *Agrobacterium*.

The binary vector with aninsert recovered by the mini-prep method from *E. coli* is introduced by electroporation into the competent cells of *Agrobacterium*. The competent cells with the introduced vector are cultured under the same conditions as normal but on medium containing hygromycin B (50 μg/ml) and kanamycin (50 μg/ml) to select transformants.

(ii) Triparent method

E. coli cells having the binary vector as described in (2) above, *E. coli* cells carrying a helper plasmid, and *Agrobacterium* cells are mixed and co-cultured to transfer the desired binary vector from *E. coli* to *Agrobacterium*.

genome library with a *lox* site, such as the constituents of a BAC library, into a transformable form for plants.

Up to this time, many genome libraries have been produced, and it is very useful if these libraries can be used for complementation testing of plants. The vector having *Ri ori* alone, or both *Ri ori* and *RK2 ori* as its replication origin, for example, the above-mentioned high capacity vector, is very stable in both *E. coli* and Agrobacterium, and has excellent transformability for plants. Based on a vector with such properties, the present vector with an *Ri ori* as the replication origin and a *lox* site and a par C site in T-DNA regions can be produced. Then, the vector can be introduced together with a circular plasmid, which is constituting a clone of a genome library with a *lox* site such as BAC library, into an *E. coli* strain which is engineered to express *cre* enzyme transiently under a specific condition. The resulting transformants are then subjected to selection by antibiotics of the library vector and then the binary vector. Thus, a vector in which the library component vector such as BAC which has *lox* site is integrated into *lox* site in T-DNA region of the another vector is obtained (FIG. 6A).

In the integrated vector obtained hereinabove, the large genome insert is inserted basically in the same manner as in the above-mentioned high capacity binary shuttle vector. Therefore, introduction of the integrated vector into *Agrobacterium* by using the procedure described above makes the introduction of the inserts into plant with high transformation efficiency shown in Table I possible. In order to remove the binary vector with *Ri ori* which was not integrated with the library plasmid, par C sequence is inserted near the lox site.

Accordingly, already constructed genome libraries made by vectors such as BAC or P1 having *lox* sites can be utilized for complementation test efficiently without the reconstruction of a library and using the high capacity binary vector of the present invention.

As shown in Table 1, the vector with both *Ri ori* and *RK2 ori* can efficiently transform the plants more markedly than that with *RK2 ori* alone does. As a result, the high capacity binary vector may be utilized to transform the plant recalcitrant to gene introduction.

In the vector, pBRGRZ, genes are only introduced in the form of genome fragments or with some promoter and terminator. It is convenient to have a vector with a multi-cloning site between the suitable promoter and terminator to introduce a gene from which the inherent promoter was removed or a full length cDNA to give desirable expression properties of an arbitrary gene (FIG. 6B). After insertion of the gene or cDNA, such vectors can be introduced into plants for transformation. In general, the combination of actin promoter and actin terminator is preferred to express a gene strongly. Preferably the same promoter or terminator are not employed repeatedly in a vector, because it may cause homologous recombination in a host cell.

The high transformation efficient strain of *Agrobacterium* with rec A⁻ is produced to maintain a large size plasmid made from the high capacity binary vector of the present invention.

In general, this large plasmid is stably maintained in both *E. coli* and *Agrobacterium*, because it is maintained as a single or low copy plasmid. In *E. coli*, strains with rec A⁻ which can not make genetic recombination, can easily be obtained, for example, DH10B. Also in *Agrobacterium*, some rec A⁻ strains are known. However, as for the high transformation efficient strains indispensable for transformation of plants which are difficult to transform, there are no report on rec A⁻ strains desirable for stable maintenance and transformation of a large plasmid.

Accordingly, such a high transformation efficient rec A⁻ strain is produced by site-directed mutation on rec A gene in EHA101 strain. The mutation is introduced into *Agrobacterium* by homologous recombination with the chromosomal rec A gene and transformed plasmid with rec A⁻ gene. A plasmid with a rec A⁻ gene produced by site-directed mutagenesis is transformed to a high transformation efficient *Agrobacterium* strain and then spread on a plate. After replica plate formation, the replica plate is irradiated by UV, and *Agrobacterium* cells are then cultured on the plate. Since the mutant with rec A⁻ can not grow under UV, such colonies are selected. When the mutant is used, the clone comprising a large genome fragment is more stably maintained in *Agrobacterium* cells. Such a mutant also can be selected by negative selection under UV irradiation as above after normal mutagenesis.

Examples

Hereinafter, the present invention is described in more detail by reference to examples showing construction of the high capacity binary shuttle vector of the present invention and its application to introduce human genomic DNA into rice. However, the present invention is not limited to the following examples.

Example 1

The high capacity binary shuttle vector of the present invention, pBIGRZ (shown in FIG. 2), was constructed in steps (1) to (3) as follows:

(1) Construction of pBH
    A hygromycin phosphotransferase gene (HPT, a gift from Dr. Hatsuyama, Hokkaido University) with a 35S

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promoter, a nopaline terminator but without *EcoRI* cleavage site was introduced using ligation into *EcoRI* site of the left border side (referred to as LB in FIG. 2) in T-DNA of the binary vector pBI121 to construct pBH. In FIG. 2, the 35S promoter and nopaline terminator are referred to as P35S and NPT respectively.

(2) Construction of pBHG

Intron-containing β-glucuronidase (iGUS) was introduced by using standard procedure between HPT and NPT in T-DNA in pBH obtained in (1) above to construct pBHG.

(3) Insertion of the replication origin of *Ri* plasmid

Ri ori, which is replication origin for *Ri* plasmid cloned from Rizobium bacteria was inserted by using ligation into *NotI* site of the outside of T-DNA.

(4) *lacZ* gene having a multi-cloning site including *HindIII*, *SpeI*, and *NotI* was prepared from the *lacZ* region in p*Bluescript* and introduced between NPT and iGUS to construct a plasmid, which was designated pBIGRZ.

As described above, since pBIGRZ has a cloning site in *lacZ*, it can be confirmed whether a fragment from genome and so forth has been inserted in the plasmid in a medium containing X-gal and IPTG.

Example 2

This example shows the introduction of about 40 kb human genome fragment into rice to examine whether the binary vector pBIGRZ obtained in Example 1 can actually introduce a 10 kb or larger genome fragment efficiently and stably into a monocotyledonous plant.

(1) A *NotI* site in pBIGRZ in *lacZ* was digested using a restriction enzyme, and the terminals formed were dephosphorylated. Then human genome *NotI* fragment DNAs (about 40 kb) which was inserted into 2 cosmids were inserted into the binary vector by ligation. The vector with the insert was introduced into *E. coli*.

(2) pBIGRZ vector with the introduced human genome fragment was electroporated from the *E. coli* to *Agrobacterium* as follows:

The binary vector was recovered from the *E. coli* by the mini-prep method using alkaline. The prepared binary vector was electroporated into competent cells of *Agrobacterium* (EHA101) carrying T-DNA-free helper plasmid, and a transformant was selected on a medium containing hygromycin (50 μg/ml) and kanamycin (50 μg/ml).

(3) pBIGRZ vector having the human genome fragment introduced stably into the *Agrobacterium* was introduced into rice scutellum calli as follows: The *Agrobacterium* into which the binary vector was introduced was co-cultivated with the calli at 25°C for 3 days, and the transformed calli were selectively cultured in a hygromycin medium (30-50 μg/ml). Thereafter, their regeneration into plants was prepared in a regeneration medium and then shoot formation was done in a hormone free medium to give re-differentiated plants. After the plants were acclimated, they were grown in pots in a greenhouse.

pBIGRZ1 and pBGRZ2, which has *Ri ori* with opposite direction, were used to examine whether the direction of *Ri ori* affects the stability of the plasmid in *Agrobacterium* (EHA101), transient expression rate of GUS after co-cultivation with rice calli, and regeneration rate of the re-differentiated plants in the above process.

The results are shown in Table 1.

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Table 1. Effect of *Ri ori* for the maintenance in bacteria and transformation of rice (cv. Nipponbare) with the plasmids.

plasmids	insert	maintenance in EHA101*	trangient GUS expression rate after co-culture with EHA101	Plant regeneration rate plants/ co-cultured cali
pBIGZ	Human genome cosmid 78C10			
	Eco RI 10kb fragment	+	10/12 (83 %)	N.T.
	Eco RI 30kb	±	0/17 (0 %)	0/60 (0 %)
	Not I 40kb	−	N.T.	N.T.
	Human genome cosmid 605			
	Eco RI 15kb	+	6/11 (55 %)	0/52 (0 %)
	Not I 40kb	−	N.T.	N.T.
	Lambda DNA			
	Hind III 27kb	+	13/17 (76 %)	N.T.
	Rice BL-1 genome cosmid			
	Not I 7kb	+	10/14 (71 %)	N.T.
	Not I 7.5kb	+	16/19 (84 %)	N.T.
	Not I 25kb	−	N.T.	—
pBIGRZ 1	Human genome cosmid 78C10			
	Not I 40kb	+	6/ 9 (67 %)	11/83 (13 %)
	Human genome cosmid 605			
	Not I 40kb	+	12/13 (92 %)	60/74 (81 %)
	Rice BL-1 genome cosmid			
	Not I 7.5kb	+	11/14 (79 %)	N.T.
	Not I 25kb	+	7/17 (41 %)	N.T.
pBIGRZ 2	Human genome cosmid			
	Not I 40kb	+	8/11 (73 %)	35/78 (45 %)
	Human genome cosmid			
	Not I 40kb	+	5/ 8 (63 %)	15/81 (19 %)

* +, ± means ca. 10^6, 10^4 clones of DH10B transformed/DNA prepared from 2 ml of EH101 culture.

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(4) Simultaneously, the same human genome fragment was introduced into pBIGZ (FIG. 1) and the introduction of this genome fragment into callus cells of rice was attempted in the same procedure as above. pBIGZ is a vector having the same structure as pBIGRZ except that it has only *RK2 ori* in pBl121 and does not have *Ri ori*.

The maintenance of the plasmid in *Agrobacterium* and degrees of regeneration of plants are shown in Table 1.

(5) By comparing pBIGRZ with *Ri* origin and pBIGZ without it, it was revealed that the stability of the plasmid in *Agrobacterium* and the plant regeneration rate significantly increased in the plant with pBIGRZ. In particular, the significant increase in the plant regeneration rate was observed in the plant with pBIGRZ.

(6) No significant difference due to the direction of *Ri ori* in the plasmid was observed in the maintenance of the plasmid in *Agrobacterium* and the rate of regeneration of plants.

(7) Genomic DNA was extracted from green leaves of the transformed plants and was digested with *Hind*III. This digested fragment was analyzed by Southern blotting with human genome *Not*I inserts in cosmids (H78C10, H605) used as probes. The results are shown in FIG. 3A and 3B.

In FIG. 3, panel A is the results of Southern hybridization of the R_0 generation plant transformed with H605 (about 44 kb), or with H78C10 (about 38 kb). In the reference lane in each panel, the digested fragment from the original human genome fragment with *Hind*III was electrophoresed at a single copy equivalent. Lanes 1 to 10 of each clone show the results of Southern hybridization of *Hind*III-digested DNA extracted from leaves of one of the regenerated plants. The results by Southern hybridization of the DNA fragment obtained from each transformant showed almost the same pattern as the reference. Panel B shows Southern brot analysis of 3 plant lives which were obtained from the same parent plant. In any plant life, almost single copy of the same fragment as control is harbored. Bands marked with dots are those hybridized to random marker.

As shown in FIG. 3A and 3B, it was demonstrated that one or more copies of genome fragments were introduced without rearrangement into the rice genome at 6/10 plants for human cosmid fragment H78C10 and 5/10 plants for fragment H605. Also it was demonstrated that such genome fragments are stably inherited by the offsprings.

As described above, when a large genome DNA fragment was inserted into the vector of the present invention and used for transformation of the plant, the inserted DNA fragment is stably maintained in the vector of the present invention without deletion or rearrangement and introduced efficiently into plant cells. Therefore, it was also demonstrated that the high capacity binary shuttle vector of the present invention is useful for narrowing the target region in the genome by complementing the functions of the gene of the interest with as large a genomic DNA fragment as possible.

In this example, the results of rice transformation were shown. However, based on the previous findings, dicotyledonous plants are generally easier in transformation by *Agrobacterium* than rice, so the high capacity binary vector of the present invention may be applied to various kinds of dicotyledonous plant, and by little modification of the experimental conditions, it also may apply in the wider range of crop plants including other important monocotyledonous crop plants.

Claims

1. A high capacity binary shuttle vector comprising T-DNA region, and a replication origin comprising *Ri ori*, or *Ri ori* and *RK2 ori*.

2. A high capacity binary shuttle vector according to claim 1, wherein said vector is based on a pBI system vector.

3. A high capacity binary shuttle vector according to claim 1 or 2, wherein said vector comprises a T-DNA region into which a gene with a multi-cloning site is introduced.

4. A high capacity binary shuttle vector according to claim 3, wherein said gene with a multi-cloning site is a *lacZ* gene.

5. A high capacity binary shuttle vector according to any preceding claim, wherein said vector comprises an antibiotic resistant gene inserted between a *lacZ* gene introduced in the T-DNA region and the border sequences of the T-DNA region.

6. A high capacity binary shuttle vector according to claim 5, wherein said antibiotic is hygromycin.

7. A high capacity binary shuttle vector according to any one of claims 1 to 6, wherein said vector is capable of integrating a large genome fragment.

8. A binary vector comprising a *lox* site in its T-DNA region, a par C gene, and an *Ri ori* as replication origin, wherein said vector is capable of integrating a clone plasmid of a circular genome library with a *lox* site by *cre* enzyme, in an *E. coli* or Agrobacterium host.

9. A binary vector for plant transformation comprising a multi-cloning site sandwiched between a promoter and a terminator for plants in a T-DNA region, and an *Ri ori* as replication origin.

10. A genomic library having the ability to transform plants, including monocotyledonous.

11. A genomic library having the ability to transform plants, wherein said library comprises a high capacity binary shuttle vector according to any one of claims 1 to 9.

12. Complementation method for assessing the function of a gene in a genome fragment inserted in a clone of a genome library of claim 11 comprising:

   introducing said vector into an *Agrobacterium* cell, and
   transferring said *Agrobacterium* into a plant.

13. Complementation method for assessing the function of a gene in a genome fragment inserted in a clone of a genome library comprising:

   integrating a plasmid clone of the library and a binary vector of any one of claims 1 to 9, wherein said library is constructed using a circular vector with a lox site and *E. coli* as host,
   introducing said integrated vector into an *Agrobacterium* cell, and
   transferring said *Agrobacterium* into a plant.

14. A gene obtained with the method according to claim 12 or 13.

15. A method for screening for a useful gene comprising using a high capacity binary shuttle vector of any one of claims 1 to 9.

16. A plant transformed using the binary vector according to any one of claims 1 to 9, or by introduction of a gene of claim 14.

17. A transformed plant according to claim 16, wherein said plant is seed plant or any plant.

18. A transformed plant according to claim 16 or claim 17, wherein said plant is a monocotyledonous plant.

19. A method for producing a high efficiency *Agrobacterium* strain with recA⁻ to maintain stably a binary vector with a large insert comprising:

   generating a hightransformation efficiency strain by the site-directed mutation on a recA gene of said strain to transformit to a recA⁻ strain,
   introducing a homologous recombination to the strain with a recA⁺ gene, between the transformed recA⁻ vector and recA⁺ gene in the cell,
   selecting the recombinant by making a replica of the plate on which the strains are spread, and
   irradiating the plate to screen for a clone which can not grow under UV irradiation.

20. A high transformation efficiency *Agrobacterium* strain with recA⁻, wherein said strain can maintain stably a high capacity binary vector with a large insert.
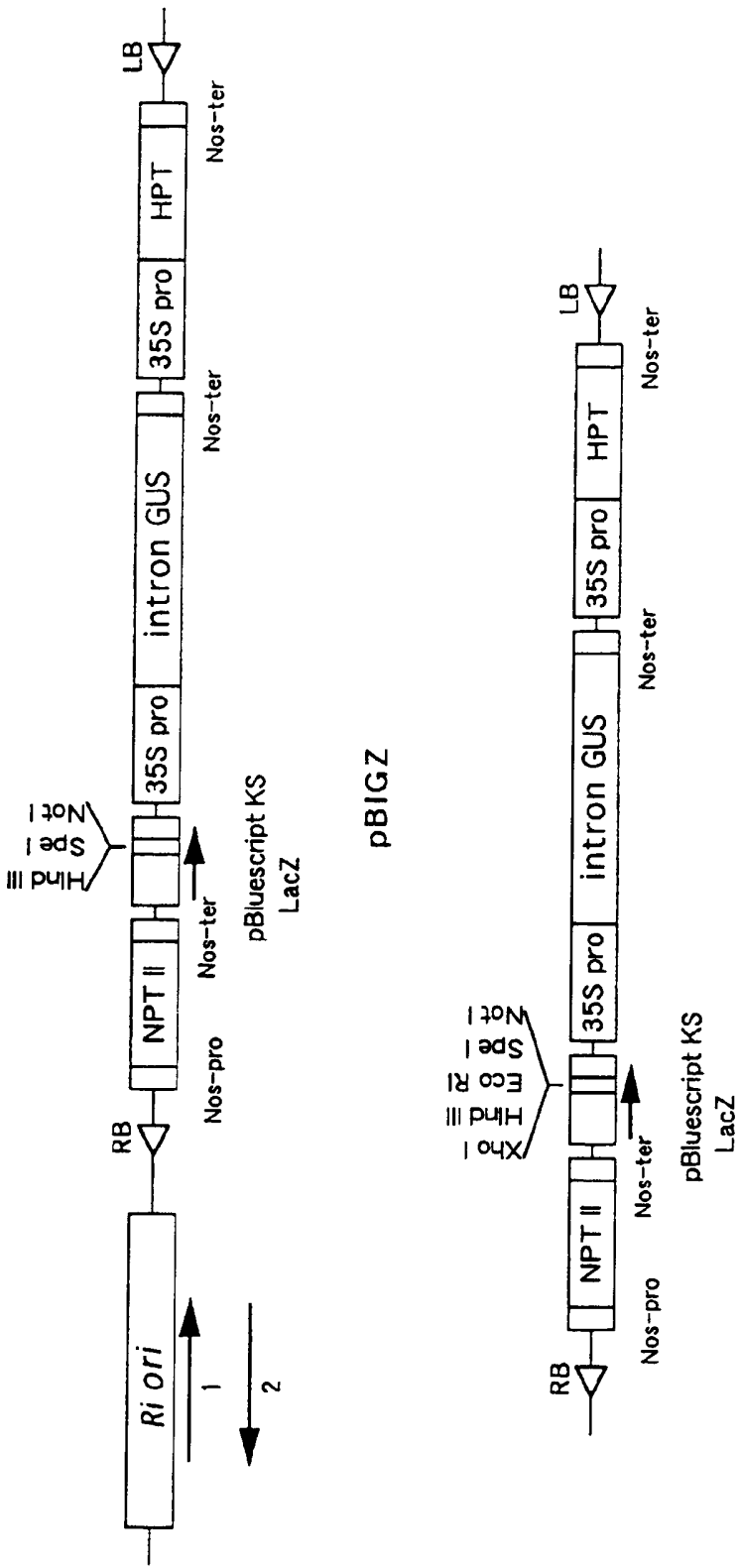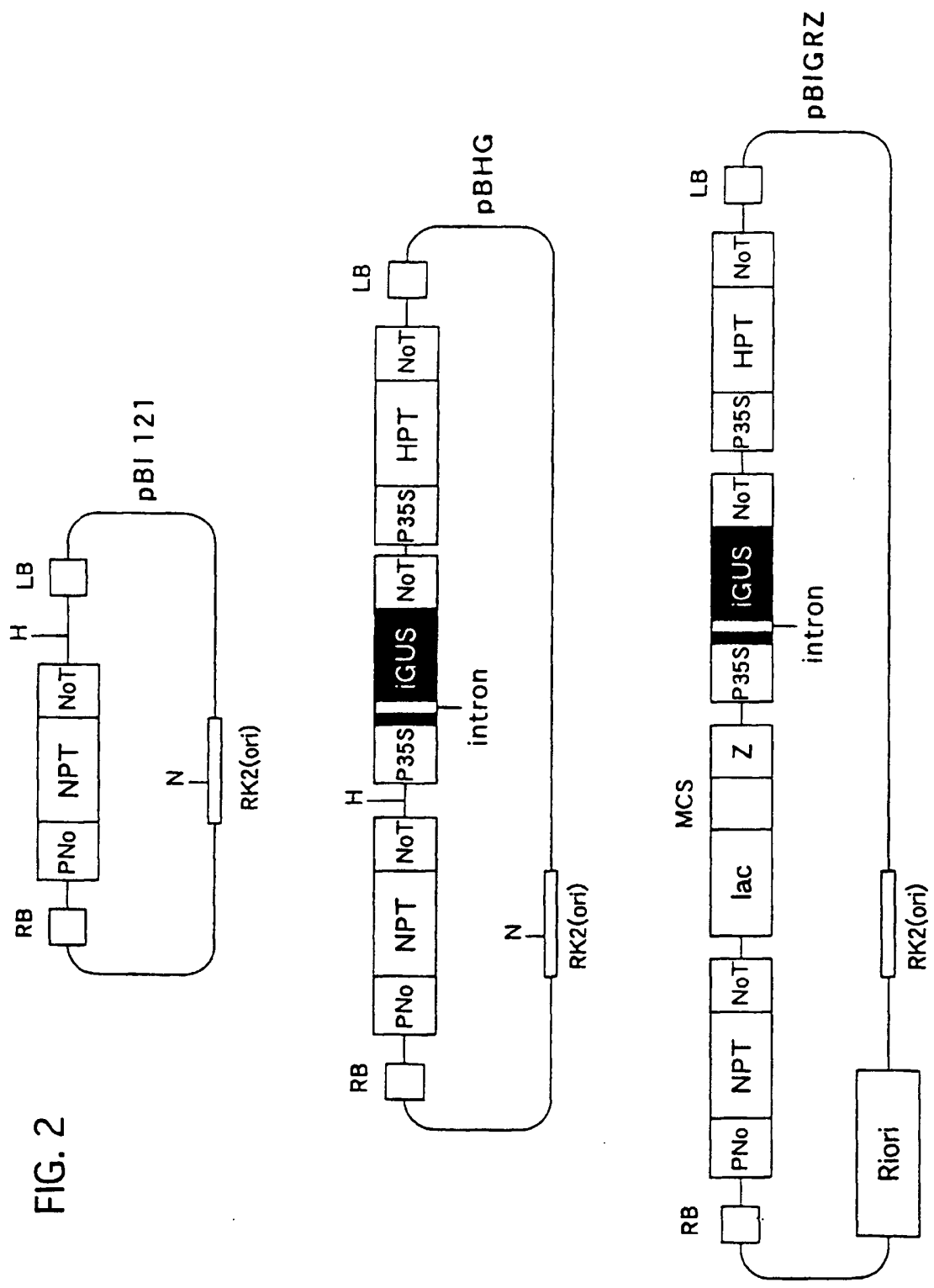
FIG. 1

1.pBIGRZ 1
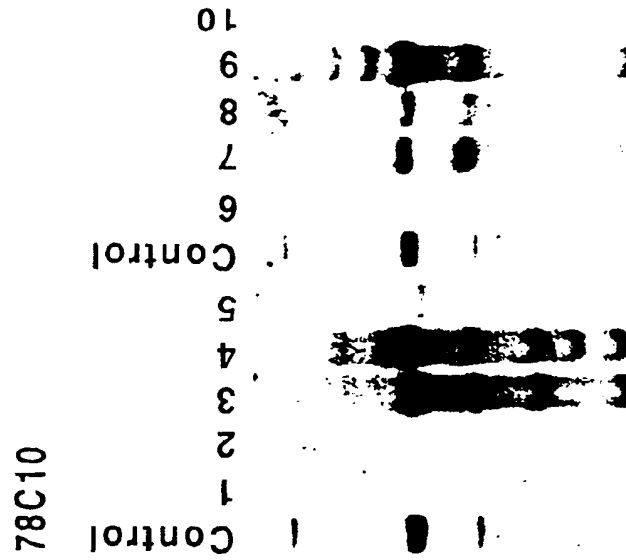2.pBIGRZ 2

pBIGZ

FIG. 2

FIG. 3B

78C10

lambda Hind III Control a-1 a-2 a-3

78C10

Control 1 2 3 4 5 Control 6 7 8 9 10

FIG. 3A

605

Control 1 2 3 4 5 Control 6 7 8 9 10

FIG. 4

FIG. 5

FIG. 6A

genome insert

BAC

cre enzyme

pBRC

FIG. 6B

pBRPT